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Title: Bioprospecting microflora in Indian coastal niches to combat biofilms.

Aim: To bioprospect microflora in the Indian coastal niches and explore their potential to combat biofilms.

Objectives:

Phase I: Standardizing biofilm formation on a suitable substratum to be used for biofilm disruption studies (3 Months)

- To form biofilms using standard biofilm forming cultures *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli K-12, Bacillus subtilis* etc.

Phase II: Screening for disruption of preformed biofilms using microcosm studies (6 months)

- To select coastal niches in Mumbai & collect samples to set up a microcosm study model
- To set up a microcosm study model & screen for biofilm disruption

Phase III: Characterization of the antibiofilm agent (6 months)

- To isolate microorganisms from the responsive niche
- To screen for the antibiofilm ability of the individual isolates
- To Characterize the agent responsible for the disruption of biofilm

Phase IV: Determination of Potency of the antibiofilm agent (4 months)

- To determine the Minimum biofilm inhibitory concentration & Minimum biofilm eradication concentration
- To evaluate the mode of action of the antibiofilm agent

Phase V: Efficacy evaluation & Applications (6 months)

- To evaluate the stability of the agent
- To evaluate the efficacy of the agent on medical implants having biofilms
- To evaluate cytotoxicity of the agent

Total estimated time for completion: 25 months

Study background & Significance of the study:

An assembly of microbial cells attached to a substratum or free floating (Desai & Ardekani, 2020), enclosed in an external matrix of primarily polysaccharides is referred to as biofilms. This assembly of cells is stringent and it is very difficult to dissociate the cells from this mat like assembly (Donlan, 2002). Bacterial biofilm formation involves four steps: i) The attachment of bacterial cells to a surface- this involves a phase of reversible attachment of cells to the surface followed by a phase of irreversible attachment brought about by the interaction between the cells and surface mediated by bacterial adhesins like lipopolysaccharide, fimbriae and other bacterial appendages, ii) This assembly of cells leads to microcolony formation iii) The cells in the assembly produce components which form the exopolysaccharide (EPS) matrix and proceeds to form a mature biofilm iv) Eventually as conditions turn unfavorable, the cells detach from the surface and move to a different niche (Crouzet et al., 2014). The independent cells which form biofilms are referred to as planktonic cells and once they are organized as a part of a biofilm assembly, then they are referred to as sessile cells. The behavior & characteristics of the sessile cells in the biofilm is found to be very different from that of their planktonic stage. This is the key in understanding the difficulty to deal with mature biofilms and clearing them from their substratum. This assemblage of cells is found on both biotic and abiotic surfaces. Their occurrence on the internal surface of industrial machines, equipment, drinking water pipes, on ship hulls have been strong reasons for huge economic and social losses (Muhammad et al., 2020). They are known to increase fluid frictional resistance on the surface of equipment, increase in the rate of corrosion, leads to decreased efficiency of production (Meesilp & Mesil, 2019).

Biofilms pose a big threat in the clinical setup. According to National Institute of Health (NIH) biofilms are the cause for 65%-80% of the bacterial infections (Lewis, 2001). This includes both medical device and non-device related infections. Data for device-related infections have been estimated for several devices, such as: 2% for breast implants; 2% for joint prostheses; 4% for mechanical heart valves; 10% for ventricular shunts; 4% for pacemakers and defibrillator, and about 40% for ventricular-assisted devices (Darouiche, 2004). They exhibit high antimicrobial resistance and also manage to evade the host immune responses as they have enhanced virulence properties and physiological mechanisms & high ability to adapt to stressful conditions. Even generally susceptible species exhibit antibiotic resistance when they are a part of biofilms due to gene transfer from resistant strains. It is challenging to treat infections caused by biofilm as the antibiotic dosage needed to kill the organisms in the biofilm is extremely high and is lethal to the host.

This enhanced resistance to antibiotics is due to multiple mechanisms possessed by cells in the biofilm which includes, "altered gene expression in biofilm-specific resistance genes (e.g., efflux pumps or exclusion of antibiotics) compared to planktonic cells, less sensitivity of most antibiotics against slower growth rate and reduced metabolic activity of cells, reduced biofilm-specific phenotype to decline the efficacy of antibiotics, degradation of antibiotics, impaired penetration of antibiotics into the biofilm matrix, stress response to hostile environmental conditions (e.g., leading to an overexpression of antimicrobial agent-destroying enzymes), altered microenvironment inside the biofilm matrix (pH, oxygen content), antimicrobial agents may be trapped and destroyed by enzymes in the biofilm matrix, altered growth rate of cells inside the biofilm, nature of antibiotics, and modification of targets" (Gebreyohannes et al., 2019).

The cells in the biofilm are protected from all external factors by the EPS but the cells manage to communicate with each other by secreting quorum sensing molecules called autoinducers. These signaling molecules are also responsible for enhancing the expression of virulence factors and for increasing the antimicrobial resistance of the cells (Tang & Zhang, 2014).

Researchers have been working on different strategies to inhibit the formation of biofilms. Different chemicals have been evaluated for their efficacy of biofilm inhibition and have been incorporated as anti biofilm coating agents during the manufacture of medical implants and devices (Yan et al., 2020). These chemicals also have to be tested for their cytotoxicity potential in order to avoid any side effects to the host. Similarly, surface of the equipment and ship hulls are coated with anti-fouling agents. But these agents have to be environmentally safe, should not pose any health hazard to other living organisms and they should not leach out into the product or into the immediate environment. It is indeed a big challenge to maintain the efficacy while not compromising on the safety (Blackman et al., 2021). Still the scientific community has managed to develop agents which can be used for effective inhibition of biofilm formation.

Along with the ability to inhibit the biofilm formation, it is necessary to have strategies to eradicate a preformed biofilm. In clinical setups, biofilms-based infections pose as a biggest challenge for therapy. There is a severe dearth in the available strategies to disperse out and kill the cells of the biofilms. The cells in biofilm form persister cells which are resistant to most of the known killing mechanisms, this makes the treatment of such infections very difficult and puts the patient's life at risk. Persister cells do not multiply rapidly, metabolically are inactive and most of the known antimicrobial mechanisms work on actively multiplying cells (Wood et al., 2013).

Several novel agents including repurposed drugs including phytochemicals, metal chelators, nanoparticles, antimicrobial peptides, microbial enzymes, bacteriophage decoctions and repurposed drugs have been evaluated for their efficacy to eliminate biofilms. Also strategies like using quorum quenching enzymes or chemicals, and playing with the expression of accessory gene regulatory (agr) system which expresses quorum sensing signals have been tested for their ability to disrupt a biofilm (Thoendel et al., 2011).

Physical methods like manual scrapping and use of jet spray have been commonly used to eliminate the biofilms. Thermal processing is a method which involved exposing biofilms to higher and lower temperatures which affects the EPS and leads to loosening of the cells. The effect of

pulsed electric fields on *P.aeruginosa* biofilm formed on medical implant devices was found to be substantial (Khan et al., 2016). But the effect was found to be enhanced when combined with antibiotics. A low temperature atmospheric pressure plasma in addition to electro-spraying was found to reduce the *E.coli* biofilm by 53-66% (Kovalova et al., 2016).

Physico-chemical methods like combining the effect of antibiotics like gentamicin or streptomycin with ultrasound and microbubbles was found to affect the matrix of *P.aeruginosa* biofilms (Ronan et al., 2016) , but it failed to eliminate the biofilm completely. While when these biofilms were exposed to ozonated water it was found that the younger biofilms exhibited higher resistance as compared to mature biofilms (Bialoszewski et al., 2011). Photodynamic therapy (PDT) was found to be effective against both biofilms and drug resistant microorganisms. This strategy involves the use of a photoactive dye which is activated on exposure to a light of specific wavelength (Konopka & Goslinski, 2007). It has been found that it could reduce 99.9% of cells in case of *P.aeruginosa* and *Methicillin resistant S.aureus* (MRSA) biofilms in a single treatment course (Biel et al., 2011).

Several of the known antibiotics have been tested for their potential to eradicate the biofilms but most of these are either partially effective or are not effective at all due to the antibiotic resistant characteristics possessed by the cells of the biofilm. It was found that when antibiotics like gentamicin was combined with L-arginine, the susceptibility of persister cells is increased to gentamicin with 99% mortality at a concentration 200*Minimum inhibitory concentration (MIC) (Lebeaux et al., 2014). This surely can pose a reason for severe side effects in the host. 0.05% caprilic acid and 0.04% glyceryl trinitrate exhibited close to 100% reduction in biofilm of *P.aeruginosa, Methicillin resistant S.aureus* (MRSA) (Rosenblatt et al., 2015).

Nanoparticles have been tested in conjugation with antibiotics to disrupt the biofilms. Chlorhexidine coated gold nanoparticles not only inhibited *K.pneumoniae* biofilms but also eliminated the pre-formed biofilms (Ahmed et al., 2016). Silver and zinc oxide nanoparticles also have been found to be effective in eradicating biofilms.

Naturally occurring compounds also have potent ability in inhibiting and eradicating biofilms. The effect of eugenol, active ingredient of clove in eradicating biofilms was evaluated. A concentration twice the MIC was effective in reducing *S.aureus* biofilm by 80-90%. It disrupts the cell to cell connections and leads to cell lysis (Yadav et al., 2015). Thyme oil, oregano oil, tea tree oil & green tea extract also have exhibited partial to complete disruption of biofilms. Moreover, a combination of caprylic acid and rhamnolipids brings about 60% reduction of biofilms by interfering with the cell to cell connections and cell substrate interactions (Díaz De Rienzo et al., 2016).

Enzymes which can degrade the components of the EPS have always been considered to be an effective resource in degrading the biofilm. Enzymes like hydrolases, amylases, proteases, DNase, trypsin have been evaluated for their efficacy in degrading the biofilm. These enzymes act on the EPD matrix and loosen up the assembly. A combinatorial approach involving enzymes and antibiotics will be a good strategy to reduce the biofilm (Pirlar et al., 2020).

Bacteriocins are promising candidates too. Nisin and lacticin Q have exhibited bactericidal activity against *S.aureus* biofilms (Okuda et al., 2013). Bacteriophages also have shown interesting ability

to penetrate the EPS layer using their polysaccharide lytic enzymes and infect the *P.aeruginosa* cells (Hughes et al., 2010).

Even though multiple strategies are available to combat biofilms, there are challenges in implementing them for therapeutic purposes. Most of the physical methods cannot be utilized due to side effects to the host. Chemical agents which are effective in disrupting biofilms can also have its own side effects and the concentration at which they are active may be hazardous to the patient. Some of the strategies tested are expensive and hence find limited applications. While others have limitations in terms of its stability or extent of use. Hence, the search for novel strategies to eliminate biofilms is still on.

It is a need of the hour to look for potential agents in natural environments. It is known that, natural components have immense potential to deal with antimicrobial resistance as it is difficult for microorganisms to gain resistance against these natural compounds. Hence it is essential to bioprospect our immediate environmental niches to look for effective, safe and stable antibiofilm agents. Bioprospecting is the exploration, extraction and screening of biological material and sometimes indigenous knowledge to discover and develop new drugs and other products (Cushnie et al., 2020). "The term bioprospecting refers to the systematic search for novel biological products and activities with biotechnological applications in natural habitats. The course of search and discovery in biotechnology starts with the selection of the most appropriate environments and sampling methods from previous information, continues with the retrieval of the biological materials and their correct storage, moves through screening for desired attributes in the form of microbial assemblages, cells, macromolecules, metabolites, or bioactive compounds using an ever-growing toolkit, and culminates with the development of a commercial product or process. This workflow represents a value-adding chain that ends with the addition of products and services that respond to society's needs" (Lozada & Dionisi, 2015).

Extreme environments are an interesting niche to explore for unique agents. Extreme environments pose intense survival challenges to microorganisms. Lack of nutrients, inappropriate temperature, pH changes, salinity, exposure to toxic chemicals & recalcitrant compounds, lack of water, presence of heavy metals etc. are conditions which microorganisms have to face often. These stressful conditions pave way for the microorganisms to evolve and adapt to these stressful environmental conditions. They develop mechanisms to metabolize and utilize the available components that too in the conditions prevalent in the environment. This opens up a new metabolic arena in this niche which can be explored for new bioactive and commercially significant compounds (Jorquera et al., 2019).

Marine microorganisms have been evolving and diversifying for billions of years in contrast microorganisms from terrestrial habitats, which possess a much shorter evolutionary history. In addition, the oceans harbor unique habitats that are mostly unexplored, making them exciting targets for bioprospecting of new microbial capabilities (Lozada & Dionisi, 2015).

Mangroves are typically tropical coastal ecosystems found in the inter-tidal zones of river deltas and back water areas. They represent highly dynamic and fragile ecosystems, yet they are the most productive and biologically diversified habitats of various life forms including plants, animals and

microorganisms. Mangroves are a resource of many different products, including; microorganisms that harbor a diverse group of industrially important enzymes, antibiotics, therapeutic proteins and vaccines; timber resistant to rot and insects; and medicinal plants (Patra et al., 2020).

Indian mangroves and their microflora have not been studied elaborately. The reports that are available hint towards the immense potential that the microflora of mangroves possess and that needs to be tapped. In this study we plan to explore the ability of the organisms in this extreme niche to combat microbial biofilms.

National Status:

The number of medical device related biofilm infections are on rise in India. Moreover, the Scoping report on Antimicrobial resistance in India published by Department of Biotechnology, Government of India in November 2017 also mentions about the potential threat of antimicrobial resistance arising from biofilm based infections. Biofilm based contamination of drinking water supplies, food and pharmaceutical products has been of increasing concern too. To tackle challenges posed due to biofilms, researchers in India have formed the India Biofilms Society in 2019 to collaborate on biofilm-based studies.

Moreover, Indian Coastal niches have immense potential in solving biothreats due of its biological diversity. Especially, the mangroves and backwater niches have been poorly explored for their microbial potential to deal with biofilms while foreign research groups have succeeded in such endeavors abroad.

International Status:

Globally too biofilms have been a big medical nuisance as it leads to severe life-threatening infections and growing incidences of antimicrobial resistance. Shipping, food, pharma industries have been worst hit because of formation of biofilms. Though several solutions are available to combat biofilms, they pose environmental toxicity, physiological toxicity or severe side effects as some unaddressed challenges. Hence, a search for effective yet safe, stable and affordable option is still a priority.

Study Hypotheses:

Microorganisms in hostile niches like coastal environments exhibit different strategies of competition due to several stress quotients. There is an opportunity in these niches to explore for antimicrobial agents which can overcome formation of biofilms and eradicate them too. If microbes in this niche have this potential, then it can be explored for biofilm management at commercial and therapeutic level.

Study Rationale:

Bioprospecting is an effective strategy to explore novel agents for combating biofilms. Marine ecosystems like sea, creeks, mangroves etc. are extreme niches where lack of nutrients, salinity,

presence of toxic pollutants and recalcitrant xenobiotics, heavy metals due to anthropogenic activities and other stressful conditions prevail. This environment poses a survival challenge for the microorganisms thriving in these niches. Along with the stressful conditions the microorganisms also have to compete with other organisms for the available resources. This antagonistic interaction and competition between microorganisms can be harnessed to look for effective antibiofilm agents in these niches. Such a possibility has not been explored widely in India and is promising strategy to solve challenges posed due to biofilm formation and the ensuing antimicrobial resistance issue. In this study, we will isolate microorganisms from coastal niches which exhibit the potential of biofilm disruption and we will characterize the agent which has the ability to eliminate a pre-formed biofilm.

Research Methodology:

1) **To form biofilms using standard biofilm forming cultures *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli K-12, Bacillus subtilis* etc.**
   The process of biofilm formation on a suitable substratum like glass using a flow system as well as stationary system will be optimized for the standard strains of *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli K-12, Bacillus subtilis* etc. (Busscher & van der Mei, 2006; Lüdecke et al., 2014)

2) **To select coastal niches in Mumbai & collect samples to set up a microcosm study model:**
   Coastal sites like sea, mangroves and creeks will be selected on the basis of accessibility and samples (Water or soil) will be collected using an appropriate sampling plan (Imchen et al., 2017). The sites will be geo tagged using GPS enabled mobile devices. The samples will be maintained in sterile containers and transported to the lab without much disturbance to the sample ambience. The conditions prevailing on the site of sample collection will be noted.

3) **To set up a microcosm study model & screen for biofilm disruption**
   Microcosm tank model will be set up and the conditions will be mimicked to that of the sample collection sites. Biofilms formed on a suitable substratum will be implanted in these tanks. These biofilms will be assessed at regular intervals of 24, 48,72 and 96hrs using microscopic methods. They will be compared with appropriate controls for proof of concept. (Han et al., 2019; Roeselers et al., 2006; Singer et al., 2006)

4) **To isolate microorganisms from the responsive niche**
   The sample niche which exhibits potential to disrupt biofilms will be further analyzed for the kind of microbial load it possesses. Microorganisms like bacteria, fungi and actinomycetes will be isolated from these samples using suitable media. (Rosmine & A.V., 2016; Saimmai et al., 2012)

5) **To screen for the antibiofilm ability of the individual isolates**

The pure isolates obtained will be grown in an appropriate broth and the metabolites present in the broth will be extracted (Mi, 2012). Their potential to eradicate preformed biofilms will be tested using assays described by (Haney et al., 2018)

6) **To Characterize the agent responsible for the disruption of biofilm**
   The agent responsible for the eradication will be characterized using hyphenated techniques like HPLC-MS or NMR etc.(Kaur et al., 2015; Rajan & Kannabiran, 2014)

7) **To determine the Minimum biofilm inhibitory concentration & Minimum biofilm eradication concentration**
   Once the agent is identified, assays to determine the MBIC & MBEC will be performed and it will be identified if the agent can also inhibit biofilm formation along with eradication.(Cruz et al., 2018; Thieme et al., 2019)

8) **To evaluate the mode of action of the antibiofilm agent**
   The mode of action of the agent will be studied by evaluating its effect on the cell integrity (Lopez-Romero et al., 2015), components of EPS (Costa Oliveira et al., 2017) , and quorum sensing molecules (Malešević et al., 2019).

9) **To evaluate the stability of the agent**
   The stability of the antibiofilm agent to different environmental conditions will be evaluated by exposing to different conditions of temperature, storage and moisture content at time intervals of 0, 7, 10,25, 50, 90 days. (de Oliveira Marreiro et al., 2014)

10) **To evaluate the efficacy of the agent on medical implants having biofilms**
    The agent will be tested for its efficacy for on-site applications like the eradication of biofilms on medical implant/devices. (Mandakhalikar et al., 2018)

11) **To evaluate cytotoxicity of the agent**
    The agent will also be evaluated for its safety for therapeutic use by subjecting it to cytotoxicity assays on selected cell lines using MTT assay. (Mohamed et al., 2018)

Statistical Plan:

A proper sampling plan will be devised to collect the samples. All experiments will be performed in triplicates and the data will be analyzed for its statistical relevance. Standard deviation & p value will be calculated for all experimental data. Statistical analysis will be done using Microsoft excel or tools like GraphPad Prism.

Limitation of the study:

Other than the above mentioned methods, integrated omics analysis including genomics, proteomics, metabolomics and interactomics can provide insights into the microbiome and their potential of a specific niche. But, given the expanse of the study and the infrastructure needed, it may be difficult to venture into omics based analysis.

Expected Outcomes:

Microorganisms will be screened for their potential of biofilm eradication. The most effective isolate will be used for production of the antibiofilm agent, the agent will be characterized and its mode of action will be elucidated. The stability, safety and application based evaluation of the agent will be performed.

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